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Role of cannabinoid receptors in hepatic fibrosis and apoptosis associated with bile duct ligation in rats

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ABSTRACT

This study assessed the effect of stimulation of CB_2 receptors or CB_1 blockade on fibrosis and apoptosis in rats subjected to bile duct ligation (BDL). It was performed in sham and BDL rats for four weeks. Fibrosis-induced rats received a CB_2 receptor agonist β-caryophyllene, CB_1 receptor antagonist, hemopressin, combination of β-caryophyllene and CB_2 antagonist, AM630 or vehicle daily during the last 2 weeks of the BDL ligation. Transaminases activity, bilirubin levels, hepatic collagen content, hydroxyproline level, Bcl2 positive hepatocytes, and mRNA expression of CB_1, CB_2 receptors and matrix metalloproteinase-1 (MMP-1) genes were measured in all animals. Bile duct ligated rats showed increased bilirubin levels, elevated transaminases activity, increased hepatic collagen content, and hydroxyproline level, reduced Bcl2 positive hepatocytes and increased expression of the assessed messengers in comparison with sham rats. However, fibrotic rats treated with either β-caryophyllene or hemopressin had reduced hepatic collagen content, improved transaminase activity and reduced bilirubin level, ameliorated CB_1 gene expression, and increased MMP-1 gene expression compared with untreated fibrotic rats. These results were associated with attenuated apoptosis with only β-caryophyllene administration. CB_2 receptor blockade by AM630 prevents the effects of β-caryophyllene on CB_1 receptor and MMP-1 genes expression. This study points out that either stimulation of CB_2 receptors or CB_1 blockade can attenuate hepatic fibrosis in bile duct ligated rats. The mechanisms underlying these incidents may open new avenues for attenuating fibrosis and apoptosis of cholestasis- induced liver diseases.

1. Introduction

The incidence of liver fibrosis has been grown dramatically in the recent years. Cholestasis is one of the most common and severe manifestations of liver diseases. Any functional disorder of the bile secretory process can cause cholestatic liver disease (Hammel et al., 2001). The major consequence of bile retention is intracellular accumulation of toxic bile constituents, consecutive cholestatic liver cell damage and finally liver fibrosis. The activation of a profibrogenic cascade of events finally leads to cirrhosis which is the major determinant of morbidity and mortality as it predisposes to hepatic failure and primary liver cancer. Therefore, hindering the progression of fibrosis to cirrhosis has been considered to be a primary goal in patients with liver disease (Friedman, 2010).

Endocannabinoids are natural lipids participating in various physiological functions, inflammatory responses, and cell proliferation (Reichenbach et al., 2012; Tam et al., 2010). They act primarily on two different types of cannabinoid receptors (CB_1 and CB_2) which mediate their various effects. Of interest, the expression of these two receptors in normal liver is low or absent (Mallat and Lotersztajn, 2008). CB_1 receptors have profibrogenic effects in liver (Teixeira-Clerc et al., 2006) and have also been implicated in the pathogenesis of alcoholic and nonalcoholic liver disease (Liu et al., 2012; Jeong et al., 2008). On the other hand, CB_2 receptor stimulation inhibits or may reverse liver fibrogenesis, shows anti-inflammatory effects in both hepatic or nonhepatic tissue (Louvet et al., 2011) and protects against liver ischemia-reperfusion injury (Horváth et al., 2012a).

β-caryophyllene is a volatile sesquiterpene and a major component (up to 35%) in the essential oil of *Cannabis sativa* L (Hendriks et al., 1975; Gertsch et al., 2008; Horváth et al., 2012b; Calleja et al., 2013). β-caryophyllene selectively binds to Δ^9 tetrahydrocannabinol binding site in the CB_2 receptor, leading to its activation and anti-inflammatory effects (Gertsch et al., 2008). It has a protective effect against liver fibrosis, inhibits hepatic

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stellate cell (HSC) activation and lipid peroxidation and has high scavenging activities against hydroxyl radical and superoxide anion (Calleja et al., 2013). It decreases the expression of fibrotic markers genes such as transforming growth factor beta-1 and tissue inhibitor of matrix metalloproteinase-1 genes (Gertsch et al., 2008).

Q2 Hemopressin is a peptide derived from hemoglobin α chain (Lippton et al. 2006). It is also a new brand from terlipressin, the drug of choice in stopping variceal bleeding in cirrhotic patients. Hemopressin acts as a CB_1 receptor-selective antagonist, as it is able to efficiently block signaling by CB_1 receptors but not by other members of G protein-coupled receptors family (including the closely related CB_2 receptors) and interacts *in vivo* with both peripheral and central pain pathways (Heimann et al., 2007).

In the present investigation, we assessed the effects of stimulation of CB_2 receptors or CB_1 blockade on fibrosis and apoptosis in rats subjected to bile duct ligation (BDL).

2. Materials and methods

2.1. Drugs and chemicals

β-caryophyllene, AM630 and hemopressin trifluoroacetate salt were purchased from Sigma-Aldrich Company (Sigma-Aldrich Co, USA). Olive oil was obtained from El-Nasr Pharmaceuticals Co. Egypt. Thiopental 500 mg IM injection was obtained from Epico Co. Egypt.

2.2. Animals

Studies were performed on 30 male adult Wistar rats (National Research Center, Cairo, Egypt) weighing 200 ± 20 g. They were housed in cages at room temperature (25 ± 2 °C) with a 12 h light/dark cycle and ambient humidity (50–60%). Rats were fed commercially available rat normal pellet diet (carbohydrates 35%, proteins 25%, lipids 7%, and vitamins 3%) and water ad libitum. They were acclimatized for one week prior to experiment. Experimental design and animal handling were approved by the Ethical Committee of the Faculty of Pharmacy, Zagazig University for Animal Use and in accordance with the guidelines of the US National Institutes of Health on animal care.

2.3. Induction of liver fibrosis

Liver fibrosis was induced by surgical ligation of the common bile duct as described previously (Uchinami et al., 2006). In brief, rats were anesthetized with thiopental (50 mg/kg, i.p) then the abdomen was shaved and disinfected. The common bile duct was exposed and twice ligated with 3-0 silk suture and sectioned between the ligatures. Sham operation was performed by gently touching the bile duct without ligation. The abdomen was closed in layers. The animals were allowed to recover on a heat pad. Rats were injected with Penicillin G (aqua-pen® vial) by deep IM injection for 3 days after surgery for prophylaxis against infection.

2.4. Experimental design

Rats were randomized into sham ($n=6$) and bile duct ligated groups (24 rats). After two weeks of bile duct ligation, rats that showed significant increase in their liver transaminases activity were selected to complete this study. They were further divided into 4 groups ($n=6$); Group I: BDL received olive oil orally; Group II: BDL received β-caryophyllene dissolved in olive oil in a dose of 5 mg/kg, p.o. (Gertsch et al., 2008); Group III: BDL received AM630 in a dose of 50 μg/rat, s.c (da Fonseca et al., 2008) thirty minutes before β-caryophyllene administration in the same previous dose; Group IV:BDL received hemopressin in a dose of 50 μg/kg, p.o. (Heimann et al., 2007). All drugs were administered daily for two weeks.

2.5. Blood and tissue sampling

At the end of the study, rats were fasted overnight. Blood samples were withdrawn from retro-orbital plexus and centrifuged at 3000 r.p.m for 30 min at 8 °C. Sera were used for determination of transaminases, total and direct bilirubin. Rats were then killed by decapitation; livers were removed, instantly washed with cold normal saline, and weighed. Each liver sample was divided into two parts. The first part was quickly frozen in liquid nitrogen and stored at −80 °C for future determination of hydroxyproline, mRNA gene expressions of cannabinoid receptors (CB_1, CB_2) and matrix metalloproteinase-1(MMP1). The other part was kept in 10% buffered formalin for further Masson trichome and immunostaining analysis.

2.6. Biochemical analyses

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total and direct bilirubin (TB, DB) were determined using commercial kits (Diamond Co, Cairo, Egypt). Hydroxyproline was determined spectrophotometrically (Chomczynski and Sacchi, 1987). For the detection of CB_1, CB_2 receptors, and MMP-1genes by Quantitative reverse transcription-polymerase chain reaction (RT-PCR), RNA was extracted using SV Total RNA isolation system (Protégé, Madison, WI, USA), reverse transcribed and amplified by PCR using RT-PCR kit (Strata gene, USA). The oligonucleotide sequences of forward and reverse primers are shown in Table 1. The amplification reactions were performed in a 50 μl final volume, with thermal cycling conditions of 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C, 30 s at 60 °C 30 s at 72 °C, and 10 min at 72 °C. Cycle threshold (Ct) data were normalized using GAPDH, used as housekeeping gene. Relative gene expression was calculated using the ΔΔCT method (Izzo et al., 2008).

$$\Delta\Delta CT = \Delta CT\,(\text{sample}) - \Delta CT\,(\text{normal})$$

where ΔCT is the difference in CT between the targeted gene and housekeeping controls by minimizing the average CT of the controls. The fold-change calculated as: $(2-\Delta\Delta CT)$.

2.7. Collagen quantification

Liver specimens were fixed using 10% buffered formalin. After proper fixation, the specimens were dehydrated in ascending grades of ethyl alcohol, cleared in xylol, impregnated and then embedded in paraffin wax. 5 μm sections were cut using rotatory microtome. Liver sections were stained with Masson trichrome

Table 1
Sequences of PCR primers.

Gene	Primer sequences
CB_1	F: 5-AGAACCTCCTCTAGGTGGGCTCG-3
	R: 5-GTACAGCGATGGCAGCTGCTG-3
CB_2	F: 5-GCAGCCTGCTGCTGACTGCTG-3
	R: 5-TGCTTTCCAGAGGACATACCC-3
MMP-1	F:5-TTGTTGCTGCCCATGAGCTT-3
	R: 5-ACTTTGTCGCCAATTCCAGG-3
GAPDH	F:5-TGCTGGTGCTGAGTATGTCG-3
	R: 5-TTGAGAGCAATGCCAGCC-3